DESCRIPTION

GLUCOSE DEHYDROGENASE β SUBUNIT AND DNA ENCODING THE SAME

Technical Field

The present invention relates to cytochrome C that constitutes a glucose dehydrogenase β subunit, to a DNA encoding the cytochrome C, and to their utilization. The glucose dehydrogenase is useful for a glucose sensor using an enzyme electrode or the like.

Background Art

Biosensors using an enzyme that specifically reacts with a particular substrate are being actively developed in various industrial fields. As for a glucose sensor, which is one of the biosensors, in particular, measurement methods and devices utilizing such methods are being actively developed mainly in medical fields. For example, the glucose sensor has a history of about 40 years since Clark and Lyons first reported about a biosensor including glucose oxidase and an oxygen electrode in combination in 1962 (L.c. Clark, J. and Lyonas, C. "Electrode systems for continuous monitoring in cardiovascular surgery." Ann. n. y. Acad. Sci., 105: 20-45).

Thus, the adoption of glucose oxidase as an enzyme for the glucose sensor has a long history. This is because glucose oxidase shows high substrate specificity for glucose and superior thermal stability, this enzyme can further be produced in a large scale, and its production cost is lower than those of other enzymes. The high substrate specificity means that this enzyme does not react with a saccharide other than glucose, and this leads to an advantage that accurate measurement can be achieved

without error in measurement values. Further, the superior thermal stability means that problems concerning denaturation of the enzyme and inactivation of its enzymatic activity due to heat can be prevented, and this leads to an advantage that accurate measurement can be performed over a long period of time.

However, although glucose oxidase has advantages as described above, it has a problem that the enzyme is affected by dissolved oxygen and this affects measurement results.

Meanwhile, in addition to glucose oxidase, a glucose sensor utilizing glucose dehydrogenase (hereinafter referred to as "glucose dehydrogenase" or "GDH") has also been developed. This enzyme is also found in microorganisms. For example, there are known glucose dehydrogenase derived from *Bacillus* (EC 1.1.1.47) and glucose dehydrogenase derived from *Cryptococcus* (EC 1.1.1.119).

The former glucose dehydrogenase (EC 1.1.1.47) is an enzyme that catalyzes a reaction of β-D-glucose + NAD(P)$^+ \rightarrow$ D-δ-gluconolactone + NAD(P)H + H$^+$, and the latter glucose dehydrogenase (EC 1.1.1.119) is an enzyme that catalyzes a reaction of D-glucose + NADP$^+ \rightarrow$ D-δ-gluconolactone + NADPH + H$^+$. The aforementioned glucose dehydrogenases derived from microorganisms are already marketed.

These glucose dehydrogenases have an advantage that they are not affected by dissolved oxygen in a measurement sample. This leads to an advantage that accurate measurement can be achieved without causing errors in measurement results even when the measurement is performed in an environment in which the oxygen partial pressure is low, or a high-concentration sample requiring a large amount of oxygen is used for the measurement.

However, although conventional glucose dehydrogenase is not affected by dissolved oxygen, it has problems of poor thermal stability and substrate specificity poorer than that of glucose oxidase. For an enzyme which is used in a sensor, an enzyme that overcomes disadvantages of both of glucose oxidase and glucose dehydrogenase has been desired.

The inventors of the present invention reported results of their studies about GDH using samples collected from soil near hot springs in Sode,K., Tsugawa,W., Yamazaki,T., Watanabe,M., Ogasawara,N., and Tanaka,M., Enzyme Microb. Technol., 19, 82-85 (1996); Yamazaki,T., Tsugawa,W. and Sode,K., Appli. Biochemi. and Biotec., 77-79/0325 (1999); and Yamazaki,T., Tsugawa,W. and Sode,K., Biotec. Lett., 21, 199-202 (1999). The microorganisms in those samples produce a coenzyme-binding GDH, and the enzymologic properties such as optimum reaction temperature, thermal stability, and substrate specificity have already been clear (See the aforementioned documents). This enzyme is a hetero oligomeric enzyme that is constituted by a catalyst subunit having a high thermal resistance (α subunit), an electron transferring subunit (β subunit), and γ subunit having an unknown function, and the activity peaks thereof are observed at 45°C and 75°C, respectively. Further, the γ and α subunit genes have been cloned, and it has been clarified that the aforementioned microorganism belongs to *Burkholderia cepacia*, and the N-terminal amino acid sequence of the β subunit has been clarified (Ken Inose, Tokyo Agricultural Engineering University Master's Thesis (2001)). However, the structure of the β subunit gene has not been reported.

Disclosure of the Invention

An object of the present invention is to provide a DNA encoding a GDH β subunit of a microorganism belonging to the genus *Burkholderia* and a method of using the DNA.

The inventors of the present invention have further advanced the study on GDH of *Burkholderia cepacia* KS1 strain and were successful in isolating a DNA encoding a GDH β subunit, thereby completing the present invention.

That is, the present invention can be described as follows.

(1) A protein defined in the following (A) or (B):

(A) a protein which has at least the amino acid sequence comprising amino acids 23 to 425 of SEQ ID NO: 16;

(B) a protein which has at least the amino acid sequence comprising amino acids 23 to 425 of SEQ ID NO: 16 including substitution, deletion, insertion or addition of 1 to 20 amino acid residues.

(2) A DNA encoding a protein defined in the following (A) or (B):

(A) a protein which has at least the amino acid sequence consisting of amino acids 23 to 425 of SEQ ID NO: 16;

(B) a protein which has at least the amino acid sequence comprising amino acids 23 to 425 of SEQ ID NO: 16 including substitution, deletion, insertion or addition of 1 to 20 amino acid residues.

(3) The DNA according to item (2), in which the DNA is defined in the following (a) or (b):

(a) a DNA including the nucleotide sequnece consisting of nucleotides 187 to 1398 of SEQ ID NO: 15;

(b) a DNA which is hybridizable with the nucleotide sequnece consisting of nucleotides 187 to 1398 of SEQ ID NO: 15 under stringent conditions.

(4) The DNA according to item (3), further including the nucleotide sequnece consisting of nucleotides 121 to 187 of SEQ ID NO: 15.

(5) A recombinant vector including a DNA according to any one of items (2) to (4).

(6) A transformant transformed with a DNA according to any one of items (2) to (4) or the recombinant vector according to item (5).

(7) A method of producing a glucose dehydrogenase β subunit, including culturing the transformant according to item (6) to produce a glucose dehydrogenase β subunit as an expression product of the DNA, and collecting the produced β subunit.

(8) The DNA according to item (3) or (4), further including the nucleotide sequnece encoding an α subunit and a γ subunit of glucose dehydrogenase of *Burkholderia cepacia*.

(9) A recombinant vector including the DNA according to item (8).

(10) A transformant transformed with the DNA according to item (8) or the recombinant vector according to item (9).

(11) A method of producing a glucose dehydrogenase complex, including culturing the transformant according to item (10) to produce a glucose dehydrogenase complex as an expression product of the DNA, and collecting the produced complex.

Best Mode for Carrying out the Invention

Hereinafter, the present invention will be described in detail.

The inventors of the present invention have searched and isolated a DNA encoding a GDH β subunit of *Burkholderia cepacia* KS1 strain. The aforementioned strain

was deposited at International Patent Organism Depositary, National Institute of Advanced Industrial Science and Technology (Central 6, 1-1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan, postal code: 305-8566) on September 25, 2000 and received a microorganism accession number of FERM BP-7306. In the present specification, the DNA encoding the GDH β subunit is sometimes referred to as the DNA of the present invention, "β subunit structural gene", or simply "β subunit gene".

The inventors of the present invention have confirmed that GDH produced by *Burkholderia cepacia* KS1 strain is a polymeric protein containing an α subunit, a β subunit, and a γ subunit. The protein of the present invention corresponds to the β subunit out of these subunits. Spectrophotometric analyses for GDH indicate that the absorption wavelength of an oxidized GDH resembles the absorption wavelengths of alcohol dehydrogenase and aldehyde dehydrogenase of *Gluconobacter* sp. and *Acetobacter* sp. that are composed of dehydrogenase cytochrome complex, and this absorption is lost by heat treatment. This fact and the difference in optimum reaction temperature of the GDH between presence and absence of the β subunit as described below have suggested that the β subunit is composed of cytochrome C.

Physical and chemical properties of the above GDH are shown below.

(1) Function: the enzyme catalyzes dehydrogenation reaction of glucose.

(2) The enzyme consists of subunits showing a molecular weight of about 60 kDa and a molecular weight of about 43 kDa in SDS-polyacrylamide gel electrophoresis under a reducing condition.

(3) The enzyme shows a molecular weight of about 380 kDa

in gel filtration chromatography using TSK Gel G3000SW (Manufactured by Tosoh Corporation).

(4) Optimal reaction temperature: around 45ºC (Tris-HCl buffer, pH 8.0).

Physical and chemical properties of an α-subunit alone are shown below.

(1)' The protein has a glucose dehydrogenase activity.

(2)' The protein shows a molecular weight of about 60 kDa in SDS-polyacrylamide gel electrophoresis under a reducing condition.

(3)' Optimal reaction temperature: around 75ºC (Tris-HCl buffer, pH 8.0).

The β subunit can be obtained together with other subunits from a culture of *Burkholderia cepacia* KS1 strain by purifying a GDH complex using GDH activity as an index. The GDH activity can be measured in the same manner as in the known GDH activity measurement. Specifically, the measurement can be performed as follows. A 10 mM potassium phosphate buffer (pH 7.0) containing 594 μM 1-methoxyphenazine methosulfate (mPMS) and 5.94 μM 2,6-dichlorophenol indophenol (DCIP) are added to an enzyme sample and glucose as a substrate, and the mixture is incubated at 37°C. A change in the absorbance of DCIP at 600 nm is traced using a spectrophotometer, and the decrease rate in the absorbance is defined as an enzyme reaction rate.

In addition, since the nucleotide sequence of a gene encoding the β subunit (SEQ ID NO: 15) has been determined by the present invention, the β subunit can also be produced by expressing a DNA having the nucleotide sequence or a DNA encoding the same amino acid sequence as the amino acid sequence encoded by this DNA in a suitable host. The amino acid sequence that can be encoded by the

open reading frame (ORF) of SEQ ID NO: 15 is shown in SEQ ID NO: 16. The N-terminal amino acid sequence of the β subunit determined from the protein was identical to the amino acids 23 to 38 of SEQ ID NO: 16. Therefore, the amino acids 1 to 22 are presumed to be a signal peptide. Note that although amino acid residue 1 is described as Val in SEQ ID NOs: 15 and 16, it has a high possibility to be Met and it also has a possibility to be dropped after the translation.

Results of homology search on the aforementioned amino acid sequence by BLAST indicated overall high homologies; 65% homology with a cytochrome C subunit of oxidoreductase dehydrogenase derived form *Ralstonia solanacearum*, 48% homology with a cytochrome C subunit of sorbitol dehydrogenase derived from *Gluconobacter oxydans*, 44% homology with a cytochrome C subunit of gluconic acid dehydrogenase derived from *Eriwinia cypripedii*, and 55.7% homology on the nucleotide sequnece level or a 46.4% homology on the amino acid level with a cytochrome C subunit of 2-keto-gluconic acid dehydrogenase derived from *Pantoea citrea*. Furthermore, the amino acid sequences of these cytochromes C reserved a hem-linking motif (SEQ ID NO: 18). These facts show that the β subunit of the present invention is cytochrome C.

The β subunit of the present invention may be a protein having the amino acid sequence consisting of the amino acids 23 to 425 of SEQ ID NO: 16 including substitution, deletion, insertion, or addion of 1 to 20, preferably 1 to 10, more preferably 1 to 5 amino acid residues in the amino acid sequence so long as it can function as a GDH β subunit. Note that the term "functions as a GDH β subunit" means "functions as cytochrome C without deteriorating the enzyme activity of the GDH".

The DNA of the present invention is a DNA that encodes the aforementioned β subunit and can be obtained from, for example, *Burkholderia cepacia* KS1 strain. The DNA of the present invention has been isolated from the chromosomal DNA of *Burkholderia cepacia* KS1 strain in the course of completion of the present invention. The DNA of the present invention can be obtained, for example, by PCR using primers having nucleotide sequences of SEQ ID NOs: 13 and 14 and the chromosomal DNA of *Burkholderia cepacia* KS1 strain as a template. In addition, since the nucleotide sequence of the DNA of the present invention and the amino acid sequence encoded by the nucleotide sequence have been clarified by the present invention, the DNA of the present invention can also be obtained by performing chemical synthesis based on these sequences. Furthermore, the DNA of the present invention can be obtained from the chromosomal DNA of *Burkholderia cepacia* KS1 strain by hybridization using the oligonucleotide prepared based on the aforementioned sequences as probes. Similarly, variants can be obtained from strains other than *Burkholderia cepacia*.

The DNA of the present invention may be one encoding a protein having the amino acid sequence consisting of the amino acids 23 to 425 of SEQ ID NO: 16 or one having the amino acid sequence including substitution, deletion, insertion, or addion of 1 to 20, preferably 1 to 10, more preferably 1 to 5 amino acid residues and encoding a protein that functions as a GDH β subunit.

Specifically, the DNA of the present invention includes a DNA having the nucleotide sequnece consisting of the nucleotides 187 to 1398 of SEQ ID NO: 15. Further, the DNA of the present invention may be a DNA that hybridizes with SEQ ID NO: 15 or a probe that can be

prepared form this sequence under stringent conditions and encodes a protein that can function as a β subunit. The stringent conditions include those conditions whereby DNAs having a 70% or more, preferably 80% or more, more preferably 90% or more homology to each other hybridize, specifically conditions of 1×SSC, 0.1% SDS, and 60°C.

The GDH β subunit can be produced by culturing a transformant that harbors the DNA of the present invention or a recombinant vector containing the DNA of the present invention to produce the GDH β subunit as an expression product of the DNA, and by collecting the GDH β subunit from the microorganism cells or the culture medium. In this case, the DNA encoding the GDH β subunit of the present invention may be expressed together with a DNA encoding an α subunit or further a DNA encoding a γ subunit to produce a GDH complex. A DNA fragment that sequentially encodes the γ subunit and the α subunit can be obtained by PCR using primers having nucleotide sequences of SEQ ID NOs: 18 and 19.

Examples of the microorganism that produces the GDH β subunit or the GDH complex include: enterobacteria including *Escherichia coli*; Gram negative bacteria such as *Pseudomonas* and *Gluconobacter*; Gram positive bacteria including bacteria belonging to the genus *Bacillus* such as *Bacillus subtilis*; yeasts such as *Saccharomyces cerevisiae*; and molds such as *Aspergillus niger*. However, the microorganism is not limited to these and any microorganism may be used so long as it is a host microorganism suitable for the production of foreign proteins.

Vectors that are used for cloning or expressing the DNA of the present invention are suitably those constructed for gene recombination from plasmids or phages

that can autonomously replicate in host microorganisms.
Examples of vectors for *E. coli* include pBR322, pUC18,
pUC118, pUC19, pUC119, pTrc99A, pBluescript, or SuperCosI,
which is a cosmid. Transfer of the DNA from the vector
that has been used in cloning the DNA of the present
invention to other recombinant vectors suitable for
expression, etc. can be readily performed by recovering
the DNA of the present invention from a recombinant vector
containing the DNA of the present invention with a
restriction enzyme or by the PCR method and ligating it
with a vector fragment. Furthermore, transformation of
microorganisms with these vectors can be performed by the
competent cell method by treatment with calcium for
bacteria belonging to the genus *Escherichia*, the
protoplast method for bacteria belonging to the genus
Bacillus, the KU method or the KUR method for yeasts, and
the micromanipulation method for molds and so forth. In
addition, the electroporation method can also be used
widely.

Selection of host microorganisms based on presence
or absence of introduction of the target recombinant
vector therein may be performed by using a chemical
resistance marker of the vector contatining the target DNA
and the like. For example, a microorganism that can grow
in a selective medium based on a chemical resistance
marker and produces GDH may be selected.

As for the culture method of the transformant,
culture conditions may be selected by considering
nutritional and physiological properties of the host. In
many cases, liquid culture is performed. It is
industrially advantageous to perform aeration culture with
shaking.

As nutrients of the medium, those usually used for

culture of microorganisms may be widely used. As carbon
sources, any assimilable carbon compounds may be used, and
examples of the compounds to be used include glucose,
sucrose, lactose, maltose, lactose, molasses, pyruvic acid
and so forth. Furthermore, as nitrogen sources, any
utilizable nitrogen compounds may be used, and examples of
the compounds to be used include peptone, meat extracts,
yeast extracts, casein hydrolysates, soybean cake alkaline
extracts and so forth. In addition, phosphate, carbonate,
sulfate, salts of magnesium, calcium, potassium, iron,
manganese, zinc and so forth, particular amino acids,
particular vitamins and so forth are used as required.

Although the culture temperature can be
appropriately changed in a range in which a bacteria grows
and produces the protein of the present invention, it is
preferably about 20ºC to 42ºC. The culture time somewhat
varies depending on the conditions. However, the culture
may be completed at an appropriate time estimated to give
the maximum GDH yield, and the culture time is usually
about 12 to 72 hours. Although pH of the medium may be
appropriately changed in a range in which a bacteria grows
and produces the protein of the present invention, it is
preferably in the range of about pH 6.0 to 9.0.

The culture medium containing cells of the
microorganism producing the protein of the present
invention in the culture can be collected and utilized as
they are. However, when the protein of the present
invention exists in the culture medium, the culture medium
is usually separated into the solution containing the
protein of the present invention and microorganism cells
by filtration, centrifugation or the like in a
conventional manner and then used. When the protein of the
present invention exists in the cells, the cells are

collected from the obtained culture by means of filtration, centrifugation or the like, and then disrupted by a mechanical method or an enzymatic method such as use of lysozyme. Further, a chelating agent such as EDTA and a surfactant are added to the cell to solubilize the protein of the present invention, as required, followed by isolation and collection as an aqueous solution.

Protein may be precipitated from the thus-obtained protein-containing solution by, for example, vacuum concentration, membrane concentration, salting out with ammonium sulfate, sodium sulfate or the like, or a fractional precipitation with a hydrophilic organic solvent such as methanol, ethanol, and acetone. Further, heat treatment and isoelectric point treatment are also effective purification means. Then, purification can be performed by a suitable combination of gel filtration using an adsorbent, gel filtration agent, etc., absorption chromatography, ion exchange chromatography and affinity chromatography to obtain a purified protein of the present invention.

A purified enzyme preparation can be obtained by isolation and purification based on column chromatography. Although the purified enzyme preparation is preferably purified to such an extent that a single band is obtained in electrophoresis (SDS-PAGE), it may contain an α-subunit or a γ-subunit.

The thus-obtained purified enzyme can be powdered by, for example, lyophilization, vacuum drying, spray drying or the like and distributed.

The GDH complex consisting of a β-subunit, an α-subunit, or whenever required, a γ-subunit of the present invention, or transformants containing those, may be used for an enzyme electrode of a glucose sensor. As the

electrode, a carbon electrode, gold electrode, platinum electrode or the like may be used, and the GDH of the present invention is immobilized on the electrode. Examples of the method for immobilization include a method of using a crosslinking reagent, a method of entrapping the enzyme in a polymer matrix, a method of covering the enzyme with a dialysis membrane, methods of using a photocrosslinking polymer, conductive polymer, oxidation-reduction polymer or the like. Alternatively, the enzyme may be immobilized in a polymer or immobilized on an electrode by adsorption together with an electronic mediator of which typical examples are ferrocene and derivatives thereof, or these methods may be used in combination. Typically, the glucose dehydrogenase of the present invention is immobilized on a carbon electrode by using glutaraldehyde, and then glutaraldehyde is blocked by a treatment with a reagent having an amine group.

The glucose concentration can be measured as follows. A buffer is placed in a thermostatic cell, and a mediator is added thereto. Then, a constant temperature is maintained. As the mediator, potassium ferricyanide, phenazine methosulfate and so forth may be used. An electrode on which the enzyme of the present invention is immobilized is used as a working electrode, and a counter electrode (e.g., platinum electrode) and a reference electrode (e.g., Ag/AgCl electrode) are used. After a constant voltage is applied to the carbon electrode to obtain a steady-state current, a sample containing glucose is added thereto and the increase of the current is measured. The glucose concentration in the sample can be calculated according to a calibration curve produced by using glucose solutions having standard concentrations.

The GDH complex containing the β subunit of the

present invention may be used as a component in an assay
kit for saccharides such as glucose. Typically, the kit
includes in addition to the GDH complex, a buffer
necessary for the assay, a mediator, a standard solution
of, for example, glucose for preparing a calibration curve,
and a manual on the use of the kit. The enzyme according
to the present invention may be provided in various forms,
for example, as a freeze-dried reagent or as a solution in
a suitable stock solution.

Example

Hereinafter, the present invention will be described
specifically with reference to examples.

Reference Example 1: Isolation of a gene encoding GDH α
subunit of *Burkholderia cepacia* KS1 strain
<1> Preparation of chromosomal DNA from *Burkhorderia
cepacia* KS1 strain.

A chromosomal gene was prepared from the
Burkhorderia cepacia KS1 strain in a conventional manner.
That is, the bacterial strain was shaken overnight at 34ºC
by using a TL liquid medium (10 g of polypeptone, 1 g of
yeast extract, 5 g of NaCl, 2 g of KH_2PO_4, 5 g of glucose
in 1 L, pH 7.2). The grown cells were collected by
centrifugation. The cells were suspended in a solution
containing 10 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA,
0.5% SDS, and 100 µg/ml proteinase K and treated at 50ºC
for 6 hours. This mixture was added to an equivalent
volume of phenol-chloroform and stirred at room
temperature for 10 minutes, and then the supernatant was
collected by centrifugation. The supernatant was added to
sodium acetate to give a final concentration of 0.3 M and
overlaid with two-fold volume of ethanol to precipitate

chromosomal DNA in the intermediate layer. The DNA was taken up with a glass rod, washed with 70% ethanol, and dissolved in an appropriate amount of TE buffer to obtain a chromosomal DNA solution.

<2> Determination of N-terminus amino acid sequence of GDH α-subunit

GDH purified in the same manner as in Example 2 was concentrated by lyophilization and separated by SDS-electrophoresis using 12.5% polyacrylamide to isolate the α-subunit. The thus obtained α-subunit was transferred onto a polyvinylidene fluoride membrane, and then the N-terminus amino acid sequence was determined by using an amino acid sequencer (PPSQ-10, manufactured by Shimadzu Corporation). As a result, it was found that the enzyme contained a peptide sequence consisting of 11 residues of the amino acid numbers 2 to 12 in the amino acid sequence of SEQ ID NO: 3.

<3> Cloning of a gene encoding α-subunit

The DNA prepared in <1> (1 μg) was subjected to partial digestion with a restriction enzyme Sau3AI, followed by treatment with calf intestinal alkaline phosphatase (CIAP). Separately, SuperCosI (obtained from Stratagene), which is a cosmid, was treated with BamHI, and the DNA fragment obtained by the partial digestion of the chromosomal DNA fragment derived from the α-15 strain with Sau3AI was introduced into SuperCosI by using T4 DNA ligase. E. coli XL-1 Blue MR (obtained from Stratagene) was transformed with the obtained recombinant DNA. A transformant was selected on an LB agar medium containing 10 μg/ml neomycin and 25 μg/ml ampicillin based on neomycin resistance and ampicillin resistance, which are antibiotic resistances on SuperCosI. The obtained transformant was cultured in the LB liquid medium. These

transformant cells were collected and suspended in a reagent for measuring the GDH activity, and a clone was selected by using dehydrogenase activity for glucose as an index. As a result, one clone showing the glucose dehydrogenase activity was obtained.

<4> Subcloning

DNA fragments containing the target gene were prepared from the cosmid, SuperCosI, containing the gene encoding the α-subunit obtained in <3>. The inserted gene fragments were cleaved from the cosmid by using a restriction enzyme *Not*I. These DNA fragments were treated with a restriction enzyme *Xba*I and introduced into a plasmid pUC18 digested with *Xba*I. The *E. coli* DH5 α-MCR strain was transformed with the plasmid pUC18 containing each inserted fragment, and colonies appeared on an LB agar medium containing 50 μg/ml of ampicillin were collected. The obtained transformants were cultured in the liquid LB medium, followed by examination for the GDH activity in the cells in the same manner as in <3>. As a result, a transformant showing the GDH activity was obtained. The plasmid was extracted from this transformant, and the inserted DNA fragment was analyzed. As a result, an insert of about 8.8 kbp was confirmed. This plasmid was designated as pKS1.

<5> Determination of the nucleotide sequence

Restriction enzyme analysis of the inserted DNA fragment in pKS1 was performed and the nucleotide sequence of the fragment was determined according to the conventional method. As a result, the sequence of the DNA encoding the N-terminus amino acid sequence of the α-subunit found in <2> was confirmed in this inserted DNA fragment, and an open reading frame containing this sequence was found. The determined nucleotide sequence and

the amino acid sequence that can be encoded by this
nucleotide sequence are as shown in SEQ ID NOs: 1 and 3.
In the nucleotide sequence of SEQ NO: 1, nucleotide
sequence downstream from nucletide number 2,386 encode
amino acid sequence of SEQ NO: 4, and encode β-subunit.

Reference Example 2: Production of a GDH-α-subunit by
recomibinant *E. coli*.

Since the nucleotide sequence of the α-subunit was
determined, a vector was prepared using the aforementioned
structural gene of the α-subunit, and a transformant was
further produced using this vector.

First, a gene to be inserted into the vector was
prepared as follows.

Amplification was performed by PCR using a genome
fragment derived from the KS1 strain as a template so that
a desired restriction enzyme site is included. The
following pair of oligonucleotide primers were used in PCR.
(Forward)
5'-CCCAAGCTTGGGCCGATACCGATACGCA-3' (SEQ ID NO: 5)
(Reverse)
5'-GAGAAGCTTTCCGCACGGTCAGACTTCC-3' (SEQ ID NO: 6)

The gene amplified by PCR was digested with a
restriction enzyme *Hin*dIII and inserted into an expression
vector pFLAG-CTS (SIGMA) at its cloning site, *Hin*dIII site.
The obtained plasmid was designated as pFLAG-CTS/α.

The *E. coli* DH5αMCR strain was transformed with the
aforementioned plasmid pFLAG-CTS/α, and colonies appeared
on the LB agar medium containing 50 μg/ml of ampicillin
were collected.

Further, when the open reading frame of the pKS1
inserted fragment was searched in the upstream of the α-
subunit, a structural gene consisting of 507 nucleotides

encoding a polypeptide including 168 amino acid residues shown in SEQ ID NO: 2 (nucleotide numbers 258 to 761 in SEQ ID NO: 1) was newly found. This structural gene was considered to encode the γ-subunit.

Since it was found that the region encoding the γ-subunit existed upstream of the coding region of the α-subunit, a recombinant vector containing a gene having a polycistronic structure continuously including the γ-subunit and the α-subunit was produced, and a transformant introduced with this vector was constructed.

First, a gene to be inserted into the vector was prepared as follows.

Amplification was performed by PCR using a genome fragment derived from the KS1 strain continuously including the structural gene of the γ-subunit and the structural gene of the α-subunit as a template so that a desired restriction enzyme site is included. The following pair of oligonucleotide primers were used for PCR.
(Forward)
5'-CATGCCATGGCACACAACGACAACACT-3' (SEQ ID NO: 7)
(Reverse)
5'-CCCAAGCTTGGGTCAGACTTCCTTCTTCAGC-3' (SEQ ID NO: 8)

The 5'-terminus and the 3'-terminus of the gene amplified by PCR were digested with *Nco*I and *Hind*III, respectively, and the gene was inserted into the vector pTrc99A (Pharmacia) at its cloning site, *Nco*I/*Hind*III site. The obtained plasmid was named pTrc99A/γ+α.

The *E. coli* DH5αMCR strain was transformed with the aforementioned plasmid pTrc99A/γ+α, and colonies appeared on the LB agar medium containing 50 µg/ml of ampicillin were collected.

The α-subunit was produced using the *E. coli* DH5αMCR strain transformed with each of the aforementioned

plasmids pKS1, pFLAG-CTS/α and pTrc99A/γ+α. Each transformant was inoculated into 3 ml of the LB medium containing 50 μg/ml of ampicillin and cultured at 37ºC for 12 hours, and the cells were collected by centrifugation. The cells were disrupted by using a French press (1,500 kgf), and a membrane fraction (10 mM potassium phosphate buffer, pH 6.0) was isolated by ultracentrifugation (4ºC, 160,400 x g, 90 minutes).

Reference Example 3: Confirmation of GDH activity
First, the GDH activity in each of the aforementioned membrane fractions was confirmed. Specifically, visual determination was performed by using 10 mM potassium phosphate buffer (pH 7.0) containing 594 μM of methylphenazine methosulfate (mPMS) and 5.94 μM of 2,6-dichlorophenolindopheol (DCIP). The results are shown below. The number of + represents the degree of color change from blue to colorless.

Membrane fraction of cultured transformant transformed with pFLAG-CTS/α : +
Membrane fraction of cultured transformant transformed with pKS1 : ++
Membrane fraction of cultured transformant transformed with pTrc99A/γ+α : +++

The GDH activity of the membrane fraction of the cultured transformant transformed with pFLAG-CTS/α containing only with the α-subunit was the lowest, and the GDH activity of the membrane fraction of the cultured transformant transformed with pTrc99A/γ+α, with which a vector was efficiently constructed, was the highest.
Although the α-subunit was expressed even in the

transformant transformed with a vector using only the structural gene of the α-subunit, the α-subunit could be efficiently obtained by using a vector containing the structural gene of the γ-subunit and the structural gene of the α-subunit in combination.

Glucose was assayed using the glucose dehydrogenase of the present invention. The enzymatic activity of the glucose dehydrogenase (α-subunit) of the present invention was measured by using glucose having various concentrations. The GDH activity was measured in 10 mM potassium phosphate buffer (pH 7.0) containing 594 μM of methylphenazine methosulfate (mPMS) and 5.94 μM of 2,6-dichlorophenolindopheol (DCIP). An enzyme sample and glucose serving as a substrate were added thereto, followed by incubation at 37ºC, and change in the absorbance of DCIP at 600nm was monitored by using a spectrophotometer. The decreasing rate of the absorbance was measured as an enzymatic reaction rate. Glucose could be quantified in the range of 0.01 to 1.0 mM using the GDH of the present invention.

Example 1: Isolation of gene encoding a GDH β-subunit of *Burkhorderia cepacia* KS1 strain

<1> Searching for *Burkholderia cepacia* KS1 strain GDH β subunit

GDH β subunit gene derived from KS1 strain was searched using *Burkholderia cepacia* J2315 strain genome database of Sanger Centre (http://www.sanger.ac.uk/). Referring to a known N-terminal sequence of KS1 strain GDH β subunit (SEQ ID No: 9), there was designed an amino acid sequence (SEQ ID NO: 10) that has high homology to each cytochrome c subunit of an alcohol dehydrogenase derived from *Acetobacter* Sp. or *Gluconobacter* Sp. (Tamaki T. et

al., Biochim Bioohys Acta 1088 (2): 292-300 (1991),
Matsushita K., et al., Biosci. Biotech. Biochem., 56, 304-
310 (1992), Takemura H., et al., J Bacteriol, 175, 6857-66
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8 (1997)); a gluconate dehydrogenase derived from *Erwinia*
sp. or *Pseudomonas* sp. (Yum DY, et al., J Bacteriol, 179,
6566-72 (1997), Matsushita K. et al., J Biochem, 85, 1173-
81 (1979)); a sorbitol dehydrogenase derived from
Gluconobacter sp. (Choi, E.S., et al., FEMS Microbiol.
Lett., 125, 45-50 (1995)); and a 2-ketogluconate
dehydrogenase derived from *Erwinia* sp. or *Pantoea* sp.
(Pujol CJ et al., J Bacteriol, 182, 2230-7, (2000)).

Based on the aforementioned amino acid sequence,
gene sequences that encode amino acid sequences having
high homologies have been searched from the aforementioned
database of *Burkholderia cepacia* J2315 strain by BLAST.
Then, the obtained five sequences were searched for
homology with the C-terminal sequence of the GDH α subunit
of KS1 strain. As a result, amino acid sequences
translated from two gene fragments showed high homologies
(>90%). Each gene fragment was as short as 200 to 500 bp,
so that sequences having high homologies with these
sequences were searched from the genome database of
Burkholderia cepacia J2315 strain by BLAST and the
fragments were joined each other. As a result, a fragment
of 3,110 bp was obtained. In the obtained nucleotide
sequence, there existed an ORF that is presumed to be the
C-terminus of the GDH and an ORF that is presumed to be
cytochrome C structural gene of 1,275 bp (SEQ ID NO: 11).
The amino acid sequence encoded by the ORF is shown in SEQ
ID NO: 12. Results of comparison between the obtained
nucleotide sequence of the J2315 strain and the nucleotide
sequence of the α subunit of KS1 strain that has already

been cloned indicate that in the downside of the α subunit, the nucleotide sequence having a high homology with the nucleotide sequnece encoding the signal peptide of cytochrome C of J2315 strain is contained.

From the above, the third ORF in the cloned fragment of *Burkholderia cepacia* KS1 strain obtained in Reference Example 1 (nucleotides 2386 et seq. of SEQ ID NO: 1) is presumed to encode the β subunit. The amino acid sequence at the N-terminus of the purified β subunit corresponds to the 5 amino acid residues translated by the nucleotide sequence consisting of nucleotides 2452 to 2466 in SEQ ID NO: 1, which also suggests that the aforementioned ORF encodes the β subunit.

<2> Amplification of β subunit structural gene using inverse PCR method

(1) Culture of microorganism cell and extraction of genome

Using 5 ml of complete medium (0.5% polypepton, 0.3% yeast extract, 0.5% NaCl), KS1 strain was cultured with shaking at 37°C overnight. Genome was extracted from the obtained microorganism cells using GennomicPrep™ Cells and Tissue DNA Isolation Kit (Amersham Pharmacia Biotech). The method was performed in accordance with the attached manual. The obtained genome was subjected to phenol/chloroform treatment and precipitated with ethanol, and then dissolved in purified water.

(2) Cyclization of genome fragment

The genome extracted from the KS1 strain was digested with *Bam*HI, *Eco*RI, *Hind*III, *Sma*I, *Sac*I, and *Xho*I and the genome fragments were recovered by precipitation with ethanol. Then, 1 μg of genome digested with the restriction enzymes was subjected to ligation reaction using a DNA ligation kit (Takara Shuzo Co., Ltd.) at 16°C overnight.

(3) PCR

50 pmol of forward primer (EF1 SEQ ID NO: 13)
designed based on the nucleotide sequence in the N-
terminal signal sequence region of the GDH β subunit of
the KS1 strain, 50 pmol of reverse primer (ER1 SEQ ID NO:
14) (all the primers were synthesized by Invitrogen on
consignment), 0.5 ml of LATaq (Takara Bio Co., Ltd.), 8 μl
of dNTP solution, and 5 μl of 10×PCR buffer was added to
purified water so as to have a total volume of 50 μl, and
PCR was performed using a program temp control system PC-
801 (ASTEC). The PCR reaction was performed under the
following conditions: after 30 cycles of 94°C for 5 minutes,
98°C for 20 seconds, 62°C for 30 seconds, 72°C for 6
minutes, and 72°C for 10 minutes.

When the genome digested with a restriction enzyme
(SmaI) is used as a template, a fragment having a size of
about 2.1 kbp was confirmed on Agarose electrophoresis.

<3> Sequencing of PCR-amplified fragment

(1) TA cloning

After the aforementioned inverse PCR product was
electrophoresed on Agarose gel, the band was cut out and
purified using Gene clean II KIT (Bio101 inc.). The
fragment was ligated to pGEM-T Vector using pGEMR-T and
pGEMR-T EASY Vector Systems (Promega). E. coli DH5α was
transformed with the ligated vector, and the transformant
was cultured on an L-agar medium containing 50 μg/ml
ampicillin, 40 μg/ml X-Gal, and 0.1 μM IPTG overnight.
From the appeared colonies, white colonies were selected
and cultured in an L medium containing 50 μg/ml ampicillin
overnight, followed by extraction of plasmids from the
cells by the alkali method.

(2) Preparation of sequence sample

The obtained plasmid was treated with RNase and 0.6

volume of 20% PEG6000/2.5 M NaCl was added thereto. The mixture was left to stand on ice for 1 hour. Thereafter, the mixture was centrifuged at 15,000 rpm and 4°C for 15 minutes to obtain a pellet. The pellet was washed with 70% ethanol and dried in vacuum. The dried product was dissolved in purified water.

(3) Analysis of nucleotide sequence of DNA

The nucleotide sequence of the inserted fragment of the plasmid obtained in (2) was analyzed using ABI PRISM™ 310 Genetic Analyzer (PERKIN-ELMER Applied Biosystems). A portion of the sequence of the inserted fragment was determined from the multicloning site of the vector using M13 primer. As a result, the nucleotide sequnece containing the N-terminus of the β subunit that had been already analyzed was confirmed. Based on this sequence, primers were sequentially prepared and used to determine the nucleotide sequence of the inserted fragment. The result is shown in SEQ ID NO: 15. Further, the amino acid sequence encoded by the ORF in the nucleotide sequence is shown in SEQ ID NO: 16.

The β subunit has 425 amino acid residues in total, and from the comparison with the N-terminal amino acid sequence already obtained, 22 residues among them are considered to be a signal peptide. The molecular weight of the β subunit calculated based on the amino acid sequence is 45,276 Da and the molecular weight 42,731 Da of the portion excluding the signal peptide is substantially identical to the molecular weight 43 kDa of the GDH β subunit of the KS1 strain. In the amino acid sequence of the β subunit, linking motifs (SEQ ID NO: 18) that links with hem in cytochrome C were confirmed at 3 positions. The ORFs were located immediately downstream of the ORF of the structural gene of the α subunit, and a sequence that

is presumed to be an SD sequence existed upstream of the initiation codon.

Homology search for the obtained amino acid sequence by BLAST showed overall high homologies; a 65% homology with cytochrome C subunit of oxidoreductase dehydrogenase derived from *Ralstonia solanacearum*, a 48% homology with a cytochrome C subunit of sorbitol dehydrogenase derived from *Gluconobacter oxydans*, a 44% homology with a cytochrome C subunit of gluconic acid dehydrogenase derived from *Eriwinia cypripedii*, and a 46.4% homology on an amino acid level with a cytochrome C subunit of 2-keto-gluconic acid dehydrogenase derived from *Pantoea citrea*. Furthermore, the amino acid sequences of cytochromes C reserved a hem-linking motif (SEQ ID NO: 18).

The structural gene of the GDH β subunit of the KS1 strain has a homology of 92.0% on the nucleotide sequnece level and of 92.2% on an amino acid level with the structural gene of the GDH β subunit of the J2315 strain.

Industrial Applicability

The present invention provides the GDH β subunit of a microorganism belonging to the genus *Burkholderia* and the DNA encoding it.